09/728,670

C97C

Patent No. 6,902,733
Request for Cert. of Correction dated November 29, 2005
Attorney Docket No. 0702-001525

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patent No.	:	6,902,733 B2 Confirmation No. 5574
Inventor	:	Collen
Issued	:	June 7, 2005
Title	:	Staphylokinase Derivatives With Polyethyleneglycol
Examiner	:	Michael Pak
Customer No.	:	28289

REQUEST FOR CERTIFICATE OF CORRECTION OF PATENT
FOR PTO MISTAKE (37 C.F.R. 1.322(a))

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ATTENTION: Decision and Certificate of Correction Branch
Patent Issue Division

Sir:

In accordance with 35 U.S.C. §254, we attach hereto Form PTO/SB/44 and a copy of proof of PTO errors and request that a Certificate of Correction be issued in the above-identified patent. The following errors appear in the patent as printed:

1. Face of Patent, See Item (54) Title, "STAPHYLOKINASE DERIVATIVES WITH POLYETHYLENEGLYCOL" should read
-- STAPHYLOKINASE DERIVATIVES --
(See application as filed as well as the Official Filing Receipt mailed 01/24/2001.)

2. Column 1, Lines 1-2, Title, "STAPHYLOKINASE DERIVATIVES WITH POLYETHYLENEGLYCOL" should read
-- STAPHYLOKINASE DERIVATIVES --
(See application as filed as well as the Official Filing Receipt mailed 01/24/2001.)

3. Column 2, Line 24, "SUMMARY OF TEE INVENTION" should read
-- SUMMARY OF THE INVENTION --
(See Preliminary Amendment dated 11/29/2000, page 2.)

4. Column 3, Line 17, "(open squares, n-6)" should read -- (open squares, n=6) --
(See Preliminary Amendment dated 11/29/2000, page 2, in the "Fig. 2" paragraph.)

5. Column 3, Line 23, "Squares: single amine acid" should read
-- Squares: single amino acid --
(See Preliminary Amendment dated 11/29/2000, page 2, second to last line.)

6. Column 3, Line 30, "(◦): 40°C" should read -- (◦): 4°C --
(See Preliminary Amendment dated 11/29/2000, page 3, Line 4.)

7. Column 3, Line 30, "(V): 37" should read -- (∇): 37 --
(See Preliminary Amendment dated 11/29/2000, page 3, Line 4.)

8. Column 3, Line 34, "following nitra-arterial" should read
-- following intra-arterial --
(See Preliminary Amendment dated 11/29/2000, page 3, Line 6.)

9. Column 6, line 7, after "ID NO: 2).", insert the following:
-- The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of *pMEX602sakB*. --
(See application as filed, page 9, Lines 17-24.

10. Column 10, Line 5, "R46177A" should read -- R77A --
(See application as filed, page 16, Line 11.)

11. Column 11, Line 52, "Qiager" should read -- Qiagen --
(See application as filed, page 19, Line 1.)

12. Column 12, Line 1, "SakSTAR(187A)" should read -- SakSTAR(I87A) --
(See application as filed, page 19, Line 11.)

13. Column 12, Line 5, "tmplate" should read -- template --.
(See application as filed, page 19, Line 14.)

14. Column 12, Line 54, "(5' CAAACAGCCAAGCTTCATTCATTCAC)" should read
-- (5' CAAACAGCCAAGCTTCATTCATTCAGC) --
(See application as filed, page 20, Line 17. The "G" before the last "C" was missing.)

15. Column 12, Line 61, "HindII" should read -- HindIII --
(See application as filed, page 20, Line 21.)

16. Column 13, Line 10, "at 0C" should read -- at 0°C --
(See application as filed, page 21, Line 4.)

17. Column 13, Line 60, "with >3" should read -- with ≥3 --
(See application as filed, page 22, Line 11.) (PTO Error)

18. Column 13, Line 62, "with >3" should read -- with ≥3 --
(See application as filed, page 22, Line 12.)

19. Column 17, Line 60, "Thrombolvtic Efficacy" should read -- Thrombolytic Efficacy --
(See application as filed, page 28, Line 27.)

20. Column 18, Line 57, "defied as neutralizing" should read -- defined as neutralizing --
(See application as filed, page 30, Line 12.)

21. Column 18, Line 59, "SakSTAR (K74Q80A)" should read -- SakSTAR (K74QE80A) --
(See application as filed, page 30, Line 13.)

22. Column 20, delete the paragraph beginning at Line 2 and ending at Line 36 and insert in its place:

The variants SakSTAR(K102C) and SakSTAR(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the construction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA AAG AGC ACG TTT CTT CTT TTT (forward) (SEQ ID NO: 9). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

(See Preliminary Amendment, filed November 29, 2000, pages 5-6, Replacement Paragraph for the first complete paragraph on page 32 of the original specification.)

23. Column 21, Line 57, "period was < 5%" should read -- period was ≤5% --
(See application as filed, page 34, Line 25.)

24. Column 21, Line 59, "C50)" should read -- C_{50}) --
(See application as filed, page 34, Line 26.)

25. Column 22, Line 34, "3.6 and . . . min" should read -- 3.6 and 3.0 min --
(See Supplemental Preliminary Amendment, pages 1-2, Replacement Paragraph 35, Lines 7-8.)

26. Column 22, Line 35, "0.52 and . . . mL/min" should read -- 0.52 and 0.32 mL/min --
(See Supplemental Preliminary Amendment, pages 1-2, Replacement Paragraph 35, Lines 7-8.)

27. Column 22, Line 39, "to size-eclusion" should read -- to size-exclusion --
(See Supplemental Preliminary Amendment, pages 1-2, Replacement Paragraph 35, Line 10.)

28. Column 23, Lines 5-6, delete "(Pool 10)=(Pool 40)+. . ., with r= . . . ."
and insert in its place -- (Pool 40) = 0..84 x (Pool 10), with r=0.94 and n=61. --
(See Supplemental Preliminary Amendment, page 3, Lines 3-4.)

29. Column 23, Line 15, "specific activities > 200" should read
-- specific activities ≥200 --
(See application as filed, page 37, Line 26.)

30. (Column 24, Line 50, "only . . . of the . . . patients" should read
-- only 1 of the 6 patients --
(See Supplemental Preliminary Amendment, page 3, Line 10)

31. Column 24, Lines 51-52, "of . . . %" should read -- rate with the variants --
(See Supplemental Preliminary Amendment, page 3, Lines 10-11.)

32. Column 24, Line 54, delete (p= . . . by Fisher's exact text) and insert in its place -- ($p = 0.01$ by 2×3 CHI^2 analysis --
(See Supplemental Preliminary Amendment, page 3, Line 13.)

33. Column 59, Claim 1, Line 4, "within up to" should read -- with up to --
(See Amendment of 08/26/2003, page 2, Claim 13, Line 6.
Claim 13 issued as Claim 1.)

Error number 4 is an obvious typographical error made by Applicant. A check for $100.00 is attached to cover the fee for correction of Applicant's mistake. The remaining errors are printing errors.

Respectfully submitted,

THE WEBB LAW FIRM

By _____

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PTO/SB/44 (04-05)
Approved for use through 04/30/2007. OMB 0651-0033
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,902,733 B2
APPLICATION NO. : 09/728,670
ISSUE DATE : June 7, 2005
INVENTOR : Désire José Collen

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

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NEW STAPHYLOKINASE DERIVATIVES

This invention relates to new staphylokinase derivatives with reduced immunogenicity, their identification, production and use in the treatment of arterial thrombosis and the preparation of a pharmaceutical composition for treating arterial thrombosis. More in particular it relates to the use of engineered staphylokinase derivatives for the preparation of a pharmaceutical composition for treating myocardial infarction.

Staphylokinase, a protein produced by certain strains of *Staphylococcus aureus*, which was shown to have profibrinolytic properties more than 4 decades ago (1,2) appears to constitute a potent thrombolytic agent in patients with acute myocardial infarction (3,4). The staphylokinase gene has been cloned from the bacteriophages *sak*øC (5) and *sak*42D (6) as well as from the genomic DNA (*sak*STAR) of a lysogenic *Staphylococcus aureus* strain (7). The staphylokinase gene encodes a protein of 163 amino acids, with amino acid 28 corresponding to the NH_2-terminal residue of full length mature staphylokinase (6,8,9). The mature protein sequence of the wild-type variant SakSTAR (9) is represented in Figure 1. Only four nucleotide differences were found in the coding regions of the *sak*ϕC, *sak*42D and *sak*STAR genes, one of which constituted a silent mutation (6,8,9).

In a plasma milieu, staphylokinase is able to dissolve fibrin clots without associated fibrinogen degradation (10-12). This fibrin-specificity of staphylokinase is the result of reduced inhibition by α_2-antiplasmin of plasmin.staphylokinase complex bound to fibrin, recycling of staphylokinase from the plasmin.staphylokinase complex following inhibition by α_2-antiplasmin, and prevention of the conversion of circulating plasminogen.staphylokinase to plasmin.staphylokinase by α_2-antiplasmin (13-15). In addition staphylokinase has a weak affinity for circulating but a high affinity for fibrin-bound plasminogen (16) and staphylokinase requires NH_2-terminal processing by plasmin to display its plasminogen activating potential (17). In several experimental animal models, staphylokinase appears to be equipotent to streptokinase for the dissolution of whole blood or plasma clots, but significantly more potent for the dissolution of platelet-rich or retracted thrombi (18,19).

procedure. DNA sequencing was performed using the dideoxy chain termination reaction method and the Automated Laser fluorescent A.L.F.^{TM} (Pharmacia). The chromogenic substrate (S2403) L-Pyroglutamyl-L-phenylalanyl-L-lysine-p-nitroanaline hydrochloride was purchased from Chromogenix (Belgium). ^{125}I-labeled fibrinogen was purchased from Amersham (UK). All other methods used in the present example have been previously described (22,27).

Construction of expression plasmids

The plasmids encoding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3'. The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a new primerless PCR using Taq polymerase (Boehringer Mannheim). After 7 cycles (1 min at 94°C, 1 min at 70°C), the extended product was reamplified by adding the 5' and 3' end primers (see above) to the PCR reaction and by cycling 25 times (1 min at 94°C, 1 min 55°C, 1 min at 72°C). The final product was purified, digested with EcoRI and HindIII and cloned into the corresponding sites of *pMEX602sakB*.

The plasmid encoding SakSTAR(E38A,K74A,E75A,R77A) was assembled by digestion of *pMEX602sakB* and *pMEX.SakSTAR(K35A,E38A,K74A,E75A,R77A)* with Bpm I which cuts between the codons for K35 and E38 of SakSTAR, and ligation of the required fragments. The plasmid encoding SakSTAR(K35A,K74A,E75A,R77A) was constructed by digestion of

angiographic puncture sites except for 5 patients who required transfusion (data not shown). Intracranial or visceral hemorrhage was not observed. Circulating fibrinogen, plasminogen and α2-antiplasmin levels remained essentially unchanged during infusion of the SakSTAR moieties (data not shown), confirming absolute fibrin specificity of staphylokinase at the dosages used. Significant in vivo fibrin digestion occurred as evidenced by elevation of fibrin fragment D-dimer levels. Intra-arterial heparin therapy prolonged aPTT levels to a variable extent (data not shown).

Antibody induction

Antibody-related SakSTAR-, SakSTAR(K74A)- and SakSTAR(K74A,E75A,R77A)-neutralizing activity and anti-SakSTAR, anti-SakSTAR(K74A) and anti-SakSTAR(K74A,E75A,R77A) IgG, were low at baseline and during the first week after the infusion (Figure 2). From the second week on, neutralizing activity levels increased to reach median values at 3 to 4 weeks of 20 µg SakSTAR(K74A) and 2.4 µg SakSTAR(K74A,E75A,R77A) neutralized per mL plasma in the patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), respectively, which is significantly lower than the median value of 93 µg wild-type SakSTAR neutralized per mL in the patients treated with SakSTAR ($p= 0.024$ for differences between the three groups by Kruskal-Wallis analysis and $p= 0.01$ and $p= 0.036$, respectively, for variants vs wild-type by Mann-Whitney rank sum test). The levels of anti-SakSTAR(K74A) and of anti-SakSTAR(K74A,E75A,R77A) IgG increased to median values at 3 to 4 weeks of 270 and 82 µg/mL plasma in patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A) respectively, which is significantly lower than the median value of 1800 µg anti-SakSTAR per mL plasma in the patients treated with SakSTAR (($p= 0.024$ for differences between the three groups by Kruskal-Wallis analysis and $p= 0.007$ and 0.05, respectively, for variants versus wild-type by Mann-Whitney rank sum test).

The titers of anti-SakSTAR(K74A) and of anti-SakSTAR(K74A,E75A,R77A) IgM increased from median baseline values of 1/460 and 1/410 to median values at 1 week of 1/510 and 1/450 in patients treated with SakSTAR(K74A) and SakSTAR(K74A,E75A,R77A), respectively, which was not significantly different from the median values of 1/320 at

purification kit from Qiagen (Hilden, Germany) or the BIO 101 RPM kit (Vista, CA), as recommended. Transformation-competent *E. coli* cells were prepared by the well-known calcium phosphate procedure. Nucleotide sequence determination was performed on double strand plasmid DNA with the dideoxy chain termination method, using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Polymerase chain reactions (PCR) were performed using Taq polymerase from Boehringer Mannheim (Mannheim, Germany) or Vent polymerase (New England Biolabs, Leusden, The Netherlands). The recombinant DNA methods required to construct the variants described in this example are well established (22,27).

Construction of expression plasmids

The variants SakSTAR(Y17A,F18A), SakSTAR(F104A), SakSTAR(F111A), SakSTAR(Y9A), SakSTAR(Y91A), SakSTAR(Y92A), SakSTAR(I87A), SakSTAR(I106A) and SakSTAR(I120A) were constructed with the Chameleon Double-Stranded Site-Directed Mutagenesis kit from Stratagene (La Jolla, USA), using the *pMEX.SakSTAR* vector as template, and following instructions of the supplier. The mutagenic oligonucleotides (not shown) were used in combination with the selection-primer LY34 5' CAAAACAGCCGAGCTTCATTCATTCAGC, which destroys the unique *Hin*dIII site located 3' to the staphylokinase encoding gene in *pMEX.SakSTAR* and allows to counter-select the non-mutant progeny by HindIII digestion. The deletion of the *Hin*dIII site was in most cases correlated with the presence of the desired mutation introduced by the mutagenic oligonucleotide. The variant SakSTAR(I133A), was constructed by performing a polymerase chain reaction on the *pMEX.SakSTAR* plasmid using the primer 818A located at the 5' end of the *sak*STAR gene (5' CAGGAAACAGAATTCAGGAG) and the mutagenic primer LY58 (5' TTCAGCATGCTGCAGTTATTTCTTTTCTGCAACAACCTTGG). The amplified product (30 cycles: 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C) was purified, digested with EcoRI and PstI, and ligated into the corresponding sites of *pMEXSakSTAR*.

The variants SakSTAR(I128A), SakSTAR(L127A) and SakSTAR(N126V) were constructed by performing a polymerase chain reaction using the primer 818A located at the 5' end of the *sakSTAR* gene and mutagenic primers (not shown). The amplified product (30 cycles: 1 sec at

94°C, 1 sec at 50°C, 10 sec at 72°C) was purified, digested with EcoRI and StyI, and ligated into the corresponding sites of *pMEXSakSTAR*. The variant SakSTAR(F125A) was constructed by performing two consecutive PCR reactions (30 cycles: : 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C). In the first reaction, a fragment of *pMEX.SakSTAR* was amplified with the primers 818A and a mutagenic primer. This amplified fragment was then used as template in a second PCR reaction with a mutagenic primer in order to further elongate the fragment downstream of the *Sty*I site present in the *sakSTAR* gene (corresponding to amino acids 130-131 of SakSTAR). The resulting product was digested with EcoRI and StyI, and ligated into the corresponding sites of *pMEXSakSTAR*.

The plasmids encoding all the other variants listed in Table 3 were constructed by direct PCR or by the spliced overlap extension polymerase chain reaction (SOE-PCR)(24) using *pMEX.SakSTAR* or available plasmids encoding SakSTAR variants as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

Expression and purification of SakSTAR variants

The SakSTAR variants were expressed and purified, as described below, from transformed *E. coli* grown in terrific broth (TB) medium (28). A 2 to 4 mL aliquot of an overnight saturated culture in LB medium was used to inoculate a 1 to 2 L culture in terrific broth supplemented with 100 µg/mL ampicillin. The culture was incubated with vigorous aeration

and at 30°C. After about 16 hours incubation, IPTG (200 µmol/L) was added to the culture to induce expression from the *tac* promoter. After 3 hours induction, the cells were pelleted by centrifugation at 4,000 rpm for 20 min, resuspended in 1/10 volume of 0.01 mol/L phosphate buffer pH 6-6.5 and disrupted by sonication at 0°C. The suspension was centrifuged for 20 min at 20,000 rpm and the supernatant was stored at 4°C or at -20°C until purification. The material was purified essentially as described above (Example 2): cleared cell lysates containing the SakSTAR variants were subjected to chromatography on a 1.6 x 5 cm column of SP-Sephadex, followed by chromatography on a 1.6 x 8 cm column of phenyl-Sepharose. The SakSTAR containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

Physicochemical and biochemical analysis

Protein concentrations were determined according to Bradford (29). SDS-PAGE was performed with the Phast System™ (Pharmacia, Uppsala, Sweden) using 10-15% gradient gels and Coomassie Brillant blue staining, and the specific activities of SakSTAR solutions were determined with a chromogenic substrate assay carried out in microtiter plates (as described in example 2). The specific activity of the different SakSTAR variants are summarized in Table 3.

Reactivity of SakSTAR variants with a panel of murine monoclonal antibodies

The methodology used to determine the reactivity of the SakSTAR variants with a panel of murine monoclonal antibodies was described in example 1 above. The results are summarized in Table 3 (the layout of this Table corresponds to the layout of Table 1, as described in example 1). Apparent association constants at least 10-fold lower than those of wild-type staphylokinase were considered as significant and are indicated in bold type in the table.

In order to obtain a comprehensive inventory of the properties of Ala-substitution variants of the SakSTAR molecule, 67 plasmids encoding variants with substitution of a single or two adjacent amino acids with Ala were constructed, expressed and purified. Together with the 35

charged residue to Ala-substitution variants previously described (22, and example 2), this analysis covers all residues in SakSTAR except Gly, Ala and Pro, as illustrated in Figure 3. Eight of the variants could not be obtained in purified form, primarily as a result of low expression levels, 11 variants were inactive, 56 had a reduced specific activity, and 27 had a maintained or increased specific activity (≥ 100 kHU/mg). The yields of purified material from cultures of expressed plasmids were 16 mg/L (median, 10 to 90 percentile range 4 to 41 mg/L). SDS polyacrylamide gel electrophoresis consistently showed one main band with $M_r \approx$ 16,000, usually representing ≥95% of total protein (not shown).

Substitution of K35, N95, S103 or K135 with Ala yielded variants with specific activities of ≥200 kU/mg. Substitution of W66, Y73 or E75 with Ala reduced the reactivity of the variants with ≥3 antibodies of epitope cluster I, of H43 or V45 with Ala that with 3 antibodies from epitope cluster II and of V32, K35, D82 and K130 with Ala that with ≥3 antibodies of epitope cluster III.

Absorption of antibodies, elicited in patients by treatment with SakSTAR

For the present example, the three plasma pools, as described in example 2 were used. The methodology used to evaluate the absorption with wild-type staphylokinase and with SakSTAR variants, of antibodies elicited in patients treated with SakSTAR, is described in detail in example 2. The results are summarized in Table 3. Whereas wild-type SakSTAR and most of the variants analyzed in this example absorbed more than 95% of the binding antibodies from pooled plasma of the 10 patients, incomplete absorption (<60%) was observed with SakSTAR(Y73A), and with SakSTAR(K74A). The predominant role of Lys74 for antibody recognition has been demonstrated previously (see example 2). The present results indicate that Tyr73 participates to the same major epitope as Lys74, or, alternatively, that substitution at Tyr73 may indirectly induce a structural modification of the "K74-epitope". Absorption with pooled plasma from 3 patients from which >95% of the antibodies were absorbed with SakSTAR(K35A,E38A,K74A,E75A,R77A) (Subpool C, see example 2) was nearly complete with most variants tested.

compounds were purified by chromatography on a 10 x 7 cm column of SP-Sepharose, equilibrated with 0.01 mol/L phosphate buffer, pH 6.0 and eluted with a 1 mol/L NaCl gradient (3 column volumes). The fractions containing SakSTAR variant were pooled, solid NaCl was added to a concentration of 2.5 mol/L and the material was chromatographed on a 10 x 20 cm column of phenyl-Sepharose followed by stepwise elution with 0.01 mol/L phosphate buffer, pH 6.0. The materials were desalted on a 10 x 45 cm column of Sephadex G25, concentrated by application on a 5 x 10 cm column of SP-Sepharose with stepwise elution with 1.0 mol/L NaCl and finally gel filtered on a 6 x 60 cm column of Superdex 75 equilibrated with 0.15 m NaCl, 0.01 mol/L phosphate buffer, pH 7.5 to further reduce their endotoxin content. The SakSTAR variant containing fractions were pooled, the protein concentration was adjusted to 1 mg/mL and the material sterilized by filtration through a 0.22 µm Millipore filter. The methodology used to determine specific activity, endotoxin contamination, bacterial sterility and toxicity in mice is described above and elsewhere (22). The purity of the preparation was evaluated by SDS gel electrophoresis on 10% gels to which 40 µg of compound was applied.

Out of culture volumes of 18 liters of SakSTAR variant, 840 mg of SakSTAR(K74Q,E80A,D82A,K130T,K135R) with a specific activity of 140 kHU/mg and 800 mg SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) with a specific activity of 150 were purified. The endotoxin content was <0.1 and 0.26 IU/mg. Gel filtration on HPLC revealed a single main symmetrical peak in the chromatographic range of the column, representing >98% of the eluted material (total area under the curve) (not shown). SDS gel electrophoresis of 40 µg samples revealed single main components (not shown). Preparations sterilized by filtration proved to be sterile on 3 day testing as described elsewhere (22). Intravenous bolus injection of SakSTAR variants in groups of 5 mice (3 mg/kg body weight), did not provoke any acute reaction, nor reduced weight gain within 8 days, in comparison with mice given an equal amount of saline (not shown).

Thrombolytic efficacy

Wild-type SakSTAR or the variants SakSTAR(K74Q,E80A,D82A,K130T,K135R) or SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) were administered intra-arterially at or

SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) IgG, were low at baseline and during the first week after the infusion (Figure 5). From the second week on, neutralizing activity levels increased to reach median values at 3 to 4 weeks of 9 µg SakSTAR(K74Q, E80A,D82A,K130T,K135R) and 0.5 µg SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) neutralized per mL plasma in the patients treated with the corresponding moieties, respectively, as compared to median value of 24 µg wild-type SakSTAR neutralized per mL in the 15 patients treated with SakSTAR. The levels of anti-SakSTAR(K74Q,E80A, D82A,K130T,K135R) and of anti-SakSTAR(E65D,K74R,E80A, D82A,K130T,K135R) IgG increased to median values at 3 to 4 weeks of 420 and 30 µg/mL plasma in patients treated with the corresponding moieties, respectively, as compared to a median value of 590 µg anti-SakSTAR per mL plasma in the patients treated with SakSTAR (Figure 5). The prevalence of immunization, defined as neutralizing activities in plasma after 2 to 4 weeks exceeding 5 µg/ml was 3 of 6 patients (50 percent) with SakSTAR(K74Q,E80A,D82A,K130T,K135R), 1 of 6 patients (17 percent) with SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R), as compared to 56 of 70 patients (80 percent) with SakSTAR. This difference is statistically highly significant (p= 0.01 by 2 x 3 Chi square analysis).

The antibodies induced by treatment with SakSTAR were completely absorbed by SakSTAR but incompletely by SakSTAR(K74Q,E80A,D82A,K130T,K135R) and by SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) (Table 12). Antibodies induced by treatment with SakSTAR(K74Q,E80A,D82A,K130T,K135R), detectable in 4 of the 6 patients, were completely (≥ 90 percent) absorbed by SakSTAR, by SakSTAR(K74Q,E80A, D82A,K130T,K135R) and by SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R), indicating that immunization was not due to neoepitopes generated by substitution of wild-type amino acids. Antibodies induced by treatment with SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) detectable in one patient (URB) were completely absorbed with SakSTAR(K74Q,E80A,D82A,K130T,K135R) and with SakSTAR(E65D,K74Q,E80A,D82A,K130T,K135R) but incompletely (85%) with wild-type SakSTAR, suggesting that a small fraction of the induced antibodies might be directed against a neoepitope in the variant used for infusion.

was eluted by a salt gradient (up to 1 M) in the same buffer. The dimeric SakSTAR(K109C) (>95% pure) containing fractions, localized by SDS-gel electrophoresis, were pooled for further analysis.

Chemical crosslinking of cysteine mutants of SakSTAR with polyethylene glycol

The thiol group of the cysteine mutant SakSTAR(K102C) was targeted for coupling with an activated polyethylene glycol, OPSS-PEG (Shearwater Polymers Europe, Enschede, The Netherlands). OPSS-PEG is a 5 kDa PEG molecule carrying a single activated thiol group at one end that react specifically at slightly alkaline pH with free thiols. Modification of SakSTAR(K102C) was achieved by incubating the molecule (100 µM) with a three-fold excess of SS-PEG in a 5 mM phosphate, pH 7.9 solution at room temperature. The extent of the reaction was monitored by following the release of 2-thiopyridone from OPSS-PEG at 412 nm. After reaction (about 15 min), the excess of OPSS-PEG was removed by purifying the derivatized SakSTAR(K102C-PEG) on a 1.6 x 5 cm column of SP-Sephadex as described above (see Example 2). The SakSTAR(K102C-PEG) containing fractions, localized by optical density at 280 nm, were pooled for further analysis. SDS-PAGE analysis and Coomassie blue staining confirmed that PEG crosslinking on SakSTAR(K102C) was quantitative. As shown in Table 13, the specific activity of the PEG-derivative was only marginally affected when compared to that of wild-type staphylokinase.

Fibrinolytic properties of SakSTAR variants in human plasma *in vitro*

The fibrinolytic and fibrinogenolytic properties of SakSTAR variants were determined as previously described. Dose- and time-dependent lysis of ^{125}I -fibrin labeled human plasma clots submerged in human plasma was obtained with four molecules: SakSTAR(K109C) as dimer and as monomer (after reduction and alkylation with iodoacetamide), the monomeric SakSTAR(K102C) and the PEG-derivatized SakSTAR(K102C). Spontaneous clot lysis during the experimental period was ≤ 5% (not shown). Equi-effective concentrations of test compound (causing 50% clot lysis in 2 hrs; C_{50}), determined graphically from plots of clot lysis at 2 hrs versus the concentration of plasminogen activator (not shown), were

CONCLUSION

In summary, the present experience illustrates that staphylokinase variants with markedly *reduced* antibody induction but *intact* thrombolytic potency can be generated. To our knowledge, this observation constitutes the first case in which a heterologous protein, with the use of protein engineering techniques, is rendered significantly less immunogenic in man without reducing its biological activity.

The present invention was inititated by the observation that certain "clustered charge-to-alanine" substitution variants of recombinant staphylokinase (SakSTAR variant (9)) had a reduced reactivity with antibodies induced by treatment with wild type SakSTAR (3,4) and induced less antibodies than wild type SakSTAR in patients with peripheral arterial occlusion (22,32,35). In an effort to optimize the specific activity versus antigenicity ratio, a comprehensive mutagenesis study, comprising the construction and expression of over 250 plasmids encoding SakSTAR variants, and the purification of the translation products was undertaken. The SakSTAR variants were characterized in terms of specific activity, affinity towards a panel of murine monoclonal antibodies and absorption of SakSTAR specific antibodies from pooled plasma of 10 patients treated with wild type SakSTAR and of two subpools of 3 patients each which reacted strongly (subpool B) or poorly (subpool C) with the immunodominant epitope K74,E75,R77. In a later phase, an additional pool of 40 patients treated with wild-type SakSTAR was also used for absorption studies. The values obtained with both pools were in good agreement. Linear regression analysis yielded: (Pool 10)= (Pool 40) + ..., with r= .....

Residues for site-directed mutagenesis were selected in three ways: 1) a comprehensive analysis of variants with 1 or 2 adjacent amino acids substituted with Ala; 2) analysis of the differential reactivity of the two natural variants SakSTAR and Sak42D (which corresponds to SakSTAR(S34G,G36R,H43R) and 3) surface exposure of the residues as derived from the three dimensional structure. From these analyses, SakSTAR(K35A), SakSTAR(N95A) and SakSTAR(S103A) emerged with specific activities ≥200 kU/mg, SakSTAR(W66A), SakSTAR(Y73A) and SakSTAR(E75A) with reduced reactivity with ≥3 of the 5 antibodies

UNITED STATES PATENT AND TRADEMARK OFFICE

COMMISSIONER FOR PATENTS
UNITED STATES PATENT AND TRADEMARK OFFICE
WASHINGTON, D.C. 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE RECD	ATTY DOCKET NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
09/728,670	11/30/2000	1646	710	702-001525	5	9	3

CONFIRMATION NO. 5574

FILING RECEIPT

Barbara E. Johnson
Webb Ziesenheim Logsdon Orkin & Hanson
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Pittsburgh, PA 15219

OC000000005702146

Date Mailed: 01/24/2001

Receipt is acknowledged of this nonprovisional Patent Application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Customer Service Center. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the PTO processes the reply to the Notice, the PTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Desire Jose Collen, Winksele-Herent, BELGIUM;

Continuing Data as Claimed by Applicant

THIS APPLICATION IS A DIV OF 09/020,018 02/06/1998
WHICH IS A CIP OF 08/784,971 01/16/1997 PAT 5,951,980
WHICH IS A CIP OF 08/499,092 07/06/1995 ABN
WHICH IS A CIP OF 08/371,505 01/11/1995 PAT 5,695,754

Foreign Applications

EUROPEAN PATENT OFFICE (EPO) 95200023.0 01/06/1995

If Required, Foreign Filing License Granted 01/18/2001

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

Title

Staphylokinase derivatives

Preliminary Class

514

Data entry by : MURSHID, SHAMSA **Team :** OIPE **Date:** 01/24/2001

PATENT APPLICATION
Atty. Docket No. 702-001525

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit Not Yet Assigned	:	
In re application of	:	
	:	**NEW STAPHYLOKINASE**
Desiré José COLLEN	:	**DERIVATIVES**
Serial No. Not Yet Assigned	:	
Examiner – Not Yet Assigned	:	

Pittsburgh, Pennsylvania
November 29, 2000

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION:

On page 3, after the title and before line 1, insert the following headings and paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of United States Patent Application Serial No. 09/020,018, filed February 6, 1998, which is a continuation-in-part of United States Patent Application Serial No. 08/784,971, filed January 16, 1997, now United States Patent No. 5,951,980, issued September 14, 1999, which is a continuation-in-part of United States Patent Application Serial No. 08/499,092, filed July 6, 1995, which is a continuation-in-part of United States Patent Application Serial No. 08/371,505, filed January 11, 1995, now United States Patent No. 5,695,754, issued December 9, 1997.

BACKGROUND OF THE INVENTION

1. Field of the Invention

On page 3, before line 6, insert the following heading:

2. Description of the Related Art

On page 4, before line 18, insert the following heading:

SUMMARY OF THE INVENTION

On page 5, before line 20, insert the following headings and paragraphs:

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a protein sequence of wild-type staphylokinase, SakSTAR (SEQ ID NO: 10). Numbering starts with the NH_2-terminal amino acid of mature full length staphylokinase.

Fig. 2 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (open circles, n=9), SakSTAR (K74A) (closed circles, n=11) or SakSTAR (K74A,E75A,R77A) (open squares, n-6) in patients with peripheral arterial occlusion. The data represent median values and interquartile ranges, in μg/ml.

Fig. 3 is a protein sequence of wild-type staphylokinase, SakSTAR with indicated amino acid substitutions.

Squares: single amino acid substitutions; circles: combined (2 to 3) amino acid to Ala substitutions.

Fig. 4 shows temperature stability of SakSTAR, (A); SakSTAR (K74Q, E80A, D82A, K130T, K135R), (B); SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R), (C); and SakSTAR (K35A, E65D, K74Q, E80A, D82A, K130T, K135R), (D).

(○): 4°C; (●): 20°C; (▽): 37°C; (▼): 56°C; (□): 70°C.

Fig. 5 is a time course of neutralizing activities (left panel) and specific IgG against administered agent (right panel) following intra-arterial infusion of SakSTAR (circles, n=15), SakStar (K74Q, E80A, D82A, K130T, K135R) (squares, n=6) or SakSTAR (E65D, K74R, E80A, D82A, K130T, K135R) (triangles, n=6) in patients with peripheral arterial occlusion. The data represent median values and 15-85 percentile ranges, in μg/mL.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

On page 9, please delete the first complete paragraph and insert the following replacement paragraph:

The plasmids encloding SakSTAR(K35A,E38A,K74A,E75A), SakSTAR(E38A,E75A,R77A), SakSTAR(E38A,E75A), SakSTAR(K35A,E75A,R77A), SakSTAR(K35A,E75A), SakSTAR(E80A), SakSTAR(D82A), SakSTAR(E75A,D82A), SakSTAR(K74A) and SakSTAR(E75A) were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24), using Vent DNA polymerase (New England Biolabs, Leusden, The Netherlands), and available or generated *sakSTAR* variants as template. Two fragments were amplified by PCR, the first one starting from the 5' end of the *staphylokinase* gene with primer 5'-CAGGAAACAGAATTCAGGAG-3' (SEQ ID NO: 1) to the region to be mutagenized (forward primer), the second one from the same region (backward primer) to the 3' end of the *staphylokinase* gene with primer 5'-CAAAACAGCCAAGCTTCATTCATTCAGC-3' (SEQ ID NO: 2).

fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (primers not shown). The two purified fragments were then assembled to gether in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

On page 32, please delete the first complete paragraph and insert the following replacement paragraph:

The variants SakSTAR(K102C) and SakSTAR(K109C), were constructed by the spliced overlap extension polymerase chain reaction (SOE-PCR) (24) using *pMEX.SakSTAR* encoding SakSTAR as template. Two fragments were amplified by PCR (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C), the first one starting from the 5' end (primer 818A) of the *staphylokinase* gene to the region to be mutagenized (forward primer), the second one from this same region (backward primer) to the 3' end of the gene with primer 818D (5' CAAACAGCCAAGCTTCATTCATTCAGC) (SEQ ID NO: 5). The forward and backward primers shared an overlap of around 24 bp (for the contruction of K102C: TAT GAT AAG AAT TGC AAA AAA GAA GAA (backward) (SEQ ID NO: 6) and TTC TTC TTT TTT GCA ATT CTT ATC ATA (forward) (SEQ ID NO: 7) for the construction of K109C: AAA AAG AAG AAA CGT GCT CTT TCC CTA (backward) (SEQ ID NO: 8) and TAG GGA

AAG AGC ACG TTT CTT CTT TTT (forward) (SEQ ID NO: 9). The two purified fragments were then assembled together in a second PCR reaction with the external primers 818A and 818D (30 cycles: 1 sec at 94°C, 1 sec at 50°C, 10 sec at 72°C). The amplified product from this final reaction was purified, digested with EcoRI and HindIII and ligated into the corresponding site of *pMEX.SakSTAR*. For each construction, the sequence of the variant was confirmed by sequencing the entire *Sak*STAR coding region.

IN THE CLAIMS:

Please cancel claims 1-12 and add the following new claims 13-21:

13. (New) A staphylokinase derivative having essentially the amino acid sequence as depicted in Figure 3 in which one or more encircled or boxed amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR-specific antibodies from plasma of patients treated with staphylokinase and further incorporating one or more polyethylene glycol groups.

14. (New) The staphylokinase derivative of claim 13 in which one polyethylene glycol group is coupled in position 102 thereon.

15. (New) The staphylokinase derivative of claim 13 in which the staphylokinase specific activity of the derivative is at least 50 percent that of wild type staphylokinase.

16. (New) Staphylokinase derivative SakSTAR(K35X,G36X,E65X,K74X, E80X,D82X,K102X,E108X,K109X,K121X,K130X,K135X,K136X,+137X) having the amino acid sequence as depicted in Figure 1 in which the amino acids Lys in position 35, Gly

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	09/728,670
Applicant	:	Desiré José Collen
Filed	:	November 30, 2000
Title:	:	Staphylokinase Derivatives
Group Art Unit	:	1646
Examiner:	:	Michael D. Pak
Docket No.	:	0702-001525

AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Office Action of March 26, 2003, please amend the above-identified application as follows:

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 4 of this paper.

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on August 26, 2003.

Anna Rosenstein
(Name of Person Mailing Paper)

Anna Rosenstein 8/26/2003
Signature Date

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

Claims 1-12 (cancelled)

Claim ~~13~~ (currently amended) A staphylokinase derivative ~~having essentially the~~ comprising an amino acid sequence ~~as depicted in figure 3 (~~which differs from **SEQ ID NO: 10**~~) in which one or more encircled or boxed amino acids have been replaced by another amino acid thus reducing the absorption of SakSTAR specific antibodies from plasma of patients treated with staphylokinase and further incorporating one or more polyethylene glycol groups~~ due to the modification of the sequence with up to four amino acid substitutions consonant with the coding regions of *sak*ØC or *sak*42D, further due to substitution of one or more amino acids therein with cysteine and further incorporating at least one polyethylene glycol group, thus reducing the clearance from plasma in patients and imparting a fibrinolytic or fibrinogenolytic property to the staphylokinase derivative.

Claim ~~14~~ (currently amended) The staphylokinase derivative of claim 13 in which one ~~polyethylene glycol group is coupled in position 102 thereon~~ cysteine is substituted at K102 and has a polyethylene glycol group coupled thereto.

Claim ~~15~~ (currently amended) The staphylokinase derivative of claim 13 in which the staphylokinase specific activity of the staphylokinase derivative is at least 50 percent that of wild type staphylokinase.

Claim ~~16~~ (currently amended) Staphylokinase derivatives SakSTAR(K35X, G36X, E65X, K74X, E80X, D82X, K102X, E108X, K109X, K121X, K130X, K135X, K136X, +137X) having essentially the amino acid sequence as depicted in Figure 1 **(SEQ ID NO: 10)** in which the amino acids Lys in position 35, Gly in position 36, Glu in position 65, Lys in position 74, Glu in position 80, Asp in position 82, Lys in position 102, Glu in position

PATENT APPLICATION
Serial No. 09/728,670
Atty. Docket No. 702-001525

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group Art Unit 1646 :

In re application of :

: **NEW STAPHYLOKINASE**
Desiré José COLLEN : **DERIVATIVES**

Serial No. 09/728,670 :

Examiner – Not Yet Assigned :

Pittsburgh, Pennsylvania
March 26, 2001

SUPPLEMENTAL PRELIMINARY AMENDMENT

Commissioner of Patents
Washington, D.C. 20231

Sir:

In response to the Notice to File Corrected Application Papers (Notice), dated January 24, 2001, please delete the specification in the application and substitute therefor the enclosed substitute specification. The required copy of the Notice is included with this reply. Prior to examination on the merits, please amend the above-identified application as follows:

IN THE SPECIFICATION:

On page 35, please delete the last paragraph that continues onto page 36 and insert the following replacement paragraph therefor:

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to Assistant Commissioner for Patents, Washington, D.C. 20231 on March 26, 2001.

Barbara E. Johnson, Registration No. 31,198
(Name of Registered Representative)

Barbara E. Johnson 03/26/2001
Signature Date

The disposition rate of staphylokinase-related antigen from blood following bolus injection of 100 μg/kg of the selected SakSTAR variants in groups of 4 hamsters could adequately be described by a sum of two exponential terms by graphical curve peeling (results not shown), from which the pharmacokinetic parameters t1/2α and Clp, summarized in Table 13 were derived. The pharmacokinetic parameters of dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) were markedly different from those of wild type SakSTAR. Initial plasma half-lives (t1/2(α)) were 3.6 and 3.0 min and plasma clearances (Clp) were 0.52 and 0.32 mL/min, for dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG), respectively. These results may be due to the increase of the Stokes radius of SakSTAR as a result of the dimerization or crosslinking with PEG. According to size-exclusion chromatography on Superdex50 by HPLC, dimeric SakSTAR(K109C) and SakSTAR(K102C-PEG) have apparent molecular weights of 33 kDa and 40 kDa, respectively.

On page 37, please delete the second *complete* paragraph and insert the following replacement paragraph therefor:

The present invention was initiated by the observation that certain "clustered charge-to-alanine" substitution variants of recombinant staphylokinase (SakSTAR variant (9)) had a reduced reactivity with antibodies induced by treatment with wild type SakSTAR (3,4) and induced less antibodies than wild type SakSTAR in patients with peripheral arterial occlusion (22,32,35). In an effort to optimize the specific activity versus antigenicity ratio, a comprehensive mutagenesis study, comprising the construction and expression of over 250 plasmids encoding SakSTAR variants, and the purification of the translation products was undertaken. The SakSTAR variants were characterized in terms of specific activity, affinity towards a panel of murine monoclonal antibodies and absorption of SakSTAR specific antibodies from pooled plasma of 10 patients treated with wild type SakSTAR and of two subpools of 3 patients each which reacted strongly (subpool B) or poorly (subpool C) with

the immunodominant epitope K74,E75,R77. In a later phase, an additional pool of 40 patients treated with wild-type SakSTAR was also used for absorption studies. The values obtained with both pools were in good agreement. Linear regression analysis yielded: (Pool 40) = 0.84 x (Pool 10) + 14, with r=0.94 and n=61.

On page 40, please delete the current paragraph and insert the following replacement paragraph therefor:

Intra-arterial administration of wild-type SakSTAR, SakSTAR(K74Q,E80A,D82A,K130T,K135R) or SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R) as a bolus of 2 mg followed by an infusion of 1 mg/hr in 6 patients with angiographically documented occlusion of a peripheral artery or bypass graft each, resulted in complete recanalization in 10 patients and partial recanalization in 2, without measurable systemic plasminogen activation. Following administration of wild-type or variant SakSTAR, neutralizing antibody titers and specific IgG levels remained low for one week. From the second or third week onwards, an increase of SakSTAR-neutralizing activity to $\geq$ μg/mL plasma was observed in the 3 of the 6 patients given SakSTAR(K74Q,E80A,D82A,K130T,K135R), and in only 1 of the 6 patients given SakSTAR(E65D,K74R,E80A,D82A,K130T,K135R). This immunization rate with the variants is significantly lower than the immunization rate of 80% observed in 70 patients treated with SakSTAR (p=0.01 by 2 x 3 Chi square analysis). The antibodies induced by treatment with the SakSTAR variants were completely absorbed by SakSTAR, and by the respective variants in all but one patient with measurable neutralizing antibody levels, indicating that immunization was not due to neoepitopes generated by substitution but to residual epitopes in the SakSTAR template.